International Journal of Pharmaceutics 399 (2010) 1–11

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Review

Chitosan as a carrier for targeted delivery of small interfering RNA

Walter E. Rudzinski[a,*], Tejraj M. Aminabhavi[b]

[a] Department of Chemistry and Biochemistry, Texas State University, San Marcos, TX 78666, United States
[b] Soniya Education Trust's College of Pharmacy, S.R. Nagar, Dharwad 580 002, India

ARTICLE INFO

Article history:
Received 13 March 2010
Received in revised form 29 June 2010
Accepted 16 August 2010
Available online 21 August 2010

Keywords:
siRNA
Chitosan
Targeted delivery
Nanoparticles

ABSTRACT

The recent discovery of RNA interference (RNAi) technology for gene therapy has triggered extensive research efforts for developing small interfering RNA (siRNA) loaded nanocomplexes of chitosan and its derivatives for silencing genes. Due to its large molecular weight (~13 kDa) and polyanionic nature (~40 negative phosphate groups), naked siRNA does not freely cross the cell membrane. Therefore, its efficient intracellular delivery requires suitable carriers to overcome the intrinsic, poor intracellular uptake and limited blood stability. Among viral and non-viral delivery vectors, the use of non-viral vectors such as chitosan or its derivatives is attractive, since these polymers are biodegradable, biocompatible, with low toxicity and high cationic potential. Even though much of the technology-base has been well established for targeted delivery of plasmid DNA using chitosan and its derivatives, only recently, has the technology been applied to the targeted delivery of siRNA. This review will explore the factors that are most important in enhancing transfection efficiency and cell specificity *in vitro* and *in vivo* including degree of deacetylation, molecular weight and chemical modification of chitosan, pH, and the charge ratio of chitosan to siRNA.

Contents

1. Introduction

RNA interference (RNAi) is a potent and highly specific gene-silencing phenomenon triggered by double-stranded RNA helices (Fire et al., 1998; Couzin, 2002). RNAi is a process, during which the introduction of 21–23 base pair small interfering RNA, called siRNA in cells results in the degradation of homologous mRNA and specific protein knockdown. Over the past decade, siRNA-mediated gene silencing has been heavily investigated in cancer therapy and other disorders due to the possible targeting of oncogenes (Leong, 2005; Agaard and Rossi, 2007; Takeshita and Ochiya, 2006; Deshayes et al., 2008; Zhang et al., 2007a; Akhtar and Benter, 2007; Ikeda and Taira, 2006; Gary et al., 2007; Behlke, 2006; Pack et al., 2005). Gene silencing is not unique to siRNA, but in fact, researchers have been silencing genes using single-stranded antisense oligodeoxyribonucleotides (ASO) for decades (Wacheck and Zangemeister-Wittke, 2006; Gleave and Monia, 2005). Successful applications of RNAi in therapy depend upon the effective knockdown of targeted transcripts and efficient intracellular delivery of either preformed siRNA or vector expressed siRNA.

* Corresponding author. Tel.: +1 512 245 3120; fax: +1 512 245 2374.
E-mail addresses: wr01@txstate.edu (W.E. Rudzinski), aminabhavi@yahoo.com (T.M. Aminabhavi).

0378-5173/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2010.08.022

Naked siRNA has a short half life of <1 h in human plasma and hence, circulating siRNAs are rapidly cleared through the kidneys even before reaching the infected target tissues. For successful gene silencing, siRNA should reach the cytoplasm of the target cells, but to achieve efficient transport, siRNA should be protected from degradation. Therefore, it is necessary to enhance the stability of siRNA in the blood stream by binding it to a transfection vector or carrier that may be either viral or non-viral. Viral vectors offer high transfection efficiency, but have safety concerns due to the potential mutation of a virus, recombination, oncogenic effects, and high costs; thus, these have limited use *in vivo*. Consequently, investigations have been initiated to develop safe and effective non-viral vectors including cationic lipids (Deshayes et al., 2008; Zhang et al., 2007a; Rajesh et al., 2007; Rao and Gopal, 2006a,b), cationic polymers (Pack et al., 2005) and dendrimers (Kihara et al., 2002, 2003) due to their better immunogenicity and safety compared to viral vectors.

The development of sequence-specific DNA or RNA analogs that can block the activity of selected single-stranded genetic sequences offers the possibility of rational design with high specificity, lacking in many current drug treatments for various diseases at relatively inexpensive cost. This review will address the relevance and importance of nanoparticles (NPs) or nanocomplexes prepared from chitosan (CS) and chemically modified chitosan for targeted delivery of siRNA. Chitosans are well known biopolymers with high cationic potential that can be prepared successfully as NPs of 100–200 nm size and would protect siRNA from degradation during transport, and exhibit high transfection efficiency. This review addresses key challenges involved in siRNA delivery to targeted cells using CS-based carriers. Parameters that are critical to achieve favorable tranfection efficiency such as degree of deacetylation (DD), molecular weight (MW), pH and N/P ratio (ratio of the positively charged CS to negatively charged siRNA) are discussed with representative examples. The effects of chemical modification of CS to enhance cell specificity and transfection efficiency both *in vitro* and *in vivo* are also discussed.

2. Chitosan

Cationic lipids are routinely used with great success as transfection agents in tissue culture, but their *in vivo* use by intravenous administration presents significant problems as these reagents can be quite toxic (Verma et al., 2003; Flynn et al., 2004). Polyethylenimine (PEI) has been used for many years to facilitate siRNA delivery (Jere et al., 2009a,b), but due to its toxicity and variable performance, it has not been generally accepted as a delivery tool for either antisense oligonucleotides or siRNA. On the other hand, CS and its derivatives have advantages in terms of site-specific delivery, cellular uptake and intracellular trafficking to improve the therapeutic potential of siRNA.

Chitosan is a naturally occurring polysaccharide composed of glucosamine and *N*-acetylglucosamine residues derived from partial deacetylation of chitin, which is generally obtained from crustacean shells. Chitosan has been recommended as a suitable candidate for delivering genes (Il'ina and Varlamov, 2005; Borchard, 2001; Mansouri et al., 2004) and drugs (Agnihotri et al., 2004a; Agnihotri and Aminabhavi, 2004b; Illum, 1998). Structurally, chitin is: poly(1 → 4), β-D(*N*-acetyl-2-amino-2 deoxyglucopyranose), which upon heating in alkaline media gives chitosan, whose chemical structure is shown in Fig. 1. The percentage of deacetylated primary amine groups along the CS chain determines the potential, while the positive charge density is responsible for the electrostatic interaction with negatively charged siRNA. Under normal physiological conditions, the reactive amine group is partially protonated, since the pK_a of D-glucosamine residue lies between 6.2 and 7.0. At a pH value of 4.0, the amine group of CS is fully protonated and functions as a cationic polyelectrolyte that can interact with the negatively charged siRNA.

A high DD of CS will increase its positive charge enabling a greater binding with siRNA. Therefore, the MW and DD of CS are important in developing an interaction with polyanionic siRNA. The protonated amine groups of CS neutralize the negatively charged siRNA and ultimately facilitate the transport of siRNA across cellular membranes and endocytosis into the cells. The electrostatic interaction of the amine groups of CS with the anionic mucus layer of the cell also enhances its mucoadhesive property. In addition, CS is capable of encapsulating siRNA to protect it from enzymatic degradation. Due to such favorable characteristics in addition to its biodegradability, biocompatibility, low immunogenicity and strong immune stimulatory capability, CS can be a safer candidate in gene therapy than many other non-viral vectors, such as cationic lipids and other polycationic polymers. The literature on using CS is growing exponentially and it is appropriate to compile such a data-base for the benefit of future researchers working in this area.

3. Design criteria and requirements

In the RNAi mechanism, double-stranded siRNA associates with nucleic acid protein complex to produce RNA-induced silencing complex (RISC). One of the RNA strands is selected and used to target the specific sequence in a messenger RNA (mRNA), leading to its degradation. The synthesis of protein encoded by mRNA is prevented and thus, RNAi technology can be used for studying functional genomics and human diseases. However, the clinical utility of systemic, therapeutic siRNA depends on developing safe and effective delivery devices to overcome the extracellular and intracellular barriers that inhibit efficient targeting to diseased cells.

Viral-based gene delivery systems usually give high transfection efficiencies, but there are concerns stemming from potential mutation, recombination, and oncogenic effects as well as high cost. In contrast, non-viral transfection agents, based on cationic polymers including CS, are promising because of diminished safety concerns. The first report of gene delivery to mammalian cells using cationic lipids as carriers was made in 1987 (Felgner et al., 1987). However, lipid-based gene delivery has some crucial limitations, including difficulty in reproducibly fabricating liposomes and gene-liposome complexes, and colloidal stability, especially upon systemic administration. In addition, non-specific cytotoxicity associated with cationic liposomes has been observed (Scales et al., 2006) as well as toxicity (for example, cell death) to some cell types *in vitro* and *in vivo* (Fillion and Philips, 1997).

The use of CS in siRNA therapies could overcome the major bottleneck of delivery to the desired cell, tissue or organ. Since siRNAs do not easily cross the cellular membrane due to their negative charge and size, CS-based gene carriers could neutralize charge and demonstrate significant versatility in terms of rigidity, hydrophobicity/hydrophilicity, charge density, biodegradability and molecular weight, all of which can be adjusted to achieve an optimal complexation with siRNA. Therefore, CS or CS-based carriers for gene delivery have unique advantages of systemic delivery over other cationic lipid-based carriers.

The design criteria for CS-based devices should consider inexpensive facile synthesis/purification, serum stability and efficient packaging of large siRNA as well as other issues including encapsulation efficiency, protection of siRNA from degradation, targeting to specific cell types, internalization, endolysosomal escape, nuclear transport, efficient decomposition, safety/nontoxicity and non-immunogenicity/nonpathogenicity. In most of these aspects, CS would offer greater flexibility and advantages over other delivery devices such as liposomes. Moreover, CS can be modified easily

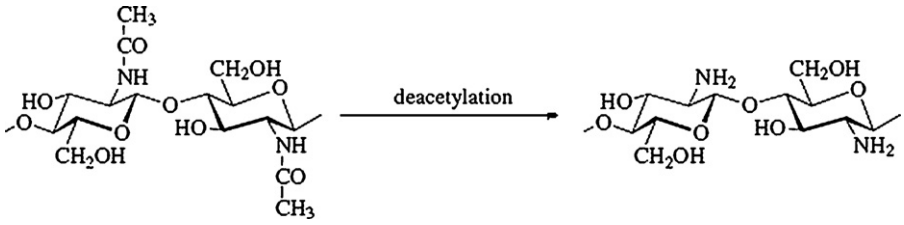

Fig. 1. Deacetylation of chitin to give chitosan.

to an appropriate size range for easy targeting. Even though the optimal size of CS/siRNA complex is debatable, most studies suggest a size of around 100 nm as a general threshold below which receptor-mediated endocytosis is operative. Further, the positive charge of CS would be an advantage for its favorable interaction with negatively charged cell membranes. Certainly, such devices have advantages to protect siRNA from nucleases and enable them to reach the cell surface without getting cleared from the system.

To achieve internalization of NPs into cells, they must reach the target cells, bind with the negatively charged cell membrane and be subsequently internalized by mechanisms specific or non-specific. To achieve gene transfer to specific cells, cell specific targeting ligands, such as monoclonal antibodies, peptides and sugars need to be conjugated to gene carriers to promote receptor-mediated endocytosis. Since CS or CS-based carriers have functional groups that are amenable to ligand conjugation, cell specific ligands can be attached. Conjugation can be executed before or after the complex formation. While the former allows for fine control of degree of substitution, the presence of ligands may interfere with siRNA complexation, since the ligand may be buried in the NP and may not be properly presented on the surface. Thus, post complexation conjugation is more advantageous because the reaction would necessarily decorate the NP surface. Specific ligands in CS would enable specific binding to the receptors of the cells to enhance transfection efficiency. The mechanism of transfection efficiency by several specific ligands such as galactose, mannose and folate in CS is well established (Vaysse et al., 2000).

Finally, the escape of NP or siRNA into the cytoplasm is a significant step in the gene transfer process and destabilization of endosomes/lysosomes by endosomolytic reagents like lipids and peptides can enhance the gene transfer efficiency. Chitosan possesses this endosomal disruptive property and a proton sponge type mechanism ensues when the nanocomplex enters the cell (Pack et al., 2005). The nanocomplex is surrounded by the endosomal membrane and because of the change in pH inside the cell; the CS becomes more protonated, which then causes diffusion of water into the endosome with a concurrent increase in osmotic pressure. Eventually, the osmotic pressure is sufficient to disrupt the membrane and cause a release of the siRNA into the cell.

Overall, it is an extremely challenging task to design CS or CS-based biopolymers incorporating all the desired features into a single structure, particularly in the absence of biological guidance. For instance, chemically stable NPs prevent siRNA release, while unstable NPs are susceptible to rapid siRNA degradation; in any case, both are undesirable in designing a suitable carrier.

4. Chitosan-based nanocarriers for gene delivery

The adaptation of controlled release technologies to DNA delivery systems has the potential to overcome extracellular barriers that limit gene therapy. Compared to traditional gene delivery systems, controlled release systems have offered improved gene delivery by maintaining a high concentration of DNA in the cellular environment and increasing the duration of transgenic expression (Luo and Saltzman, 2000). Gene silencing can be achieved either by direct administration of siRNA or by using DNA-directed RNAi that enables *in vivo* production of siRNAs. Many groups and companies are working on developing strategies to deliver siRNAs to cells in culture and in tissues in various animal models. Studies in the realm of non-viral DNA gene therapy (Fernando and Fletcher, 2006) have been ongoing for years and will continue toward improving systemic delivery and transfection efficiencies to the levels required of *in vivo* trials. Compared to antisense technology, siRNAs tend to have more non-specificity in terms of the potential of broad off-target gene modulation by a single siRNA sequence. Various studies using peptide nucleic acids have reported the expression of multiple off-target genes after the introduction of siRNA sequence into cells (Dean, 2000; Nielsen, 2001).

Numerous research groups have reported the development of CS-based nanocomplexes for the targeted delivery of plasmid DNA (pDNA) (Borchard, 2001; Garnett, 1999; Gary et al., 2007; Weecharangsan et al., 2008; Romoren et al., 2003; Sato et al., 2001). Other studies on DNA delivery with CS as a carrier have shown effective expression of reporter genes *in vitro* and *in vivo* (Kiang et al., 2004; Liu et al., 2005), This review is focused on CS-based carriers for siRNA. The concepts and protocols employed in pDNA delivery can be adapted to siRNA delivery.

In CS-based devices, it is important to consider the appropriate balance between CS and siRNA moieties in terms of N/P ratio, i.e., number of CS nitrogens (N) per siRNA phosphates (P). Adjustment of this ratio along with MW and DD of CS is crucial in order to obtain the desired particle size and electrical zeta potential. As regards the effect of pH, varying levels of interaction of CS with siRNA studied under different pH conditions (4.1–9.5) investigated by atomic force microscopy (Xu et al., 2007) indicate that pH has a role in maintaining the stability of the nanocomplex.

In view of the limited transfection capability of nascent CS, studies have also concentrated on chemically modifying the CS structure to improve transfection efficiency. Based on the earlier work on CS–pDNA complexes, researchers have developed CS conjugation with polyethylene glycol (PEG) or deoxycholic acid, trimethylation, thiolation, galactosylation, etc. (Mao et al., 2001; Florea et al., 2006; Lee et al., 2007; Park et al., 2001). PEG conjugation of CS provides enhanced particle stability with minimum aggregation without sacrificing transfection efficiency (Mao et al., 2001). PEGylation of CS elongates the plasma circulation time and prolongs gene transfer for sustained delivery of DNA (Zhang et al., 2007b). Blend microspheres of PEG grafted CS with PLGA also result in sustained release of DNA for up to nine weeks (Yun et al., 2005). Conjugation of deoxycholic acid to CS provides a greater formation of self aggregated nanocomplexes with pDNA (Lee et al., 2007) while trimethylation of CS enhances its solubility at physiological pH (Florea et al., 2006). Thiolated CS shows significant enhancement of transfection when compared to the commercial transfection reagent, lipofectin (Lee et al., 2007). Finally, conjugation of galactose with CS demonstrates enhanced targeting to liver (Park et al., 2001). In any case, the reported literature on pDNA delivery offers novel approaches for siRNA delivery using CS as a vector (Gao et al., 2005).

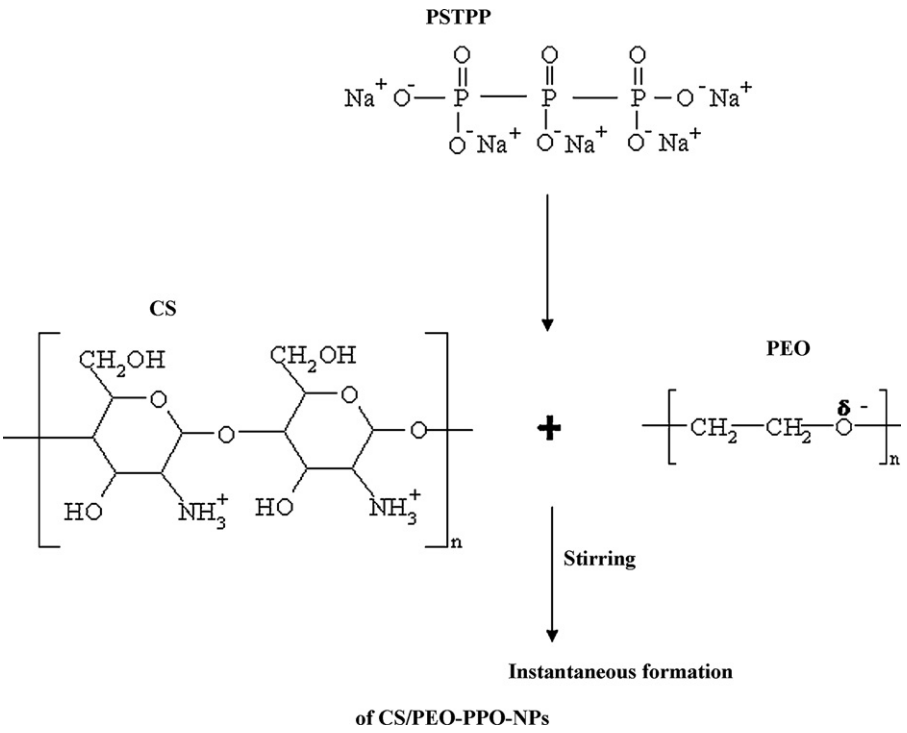

Fig. 2. Scheme for the preparation CS nanoparticles.

5. Parameters affecting transfection efficiency of chitosan-based carriers

Even though there is no general rule *per se* as to the effect of parameters like MW, DD, nitrogen/phosphorus (N/P) charge ratio of chitosan to siRNA, pH and substituent groups on the chemically modified CS, studies have elucidated the optimum conditions involving these parameters for achieving a practically useful range of transfection efficiency. The following sub-sections will discuss these in greater depth.

5.1. Molecular weight, degree of deacetylation and charge ratio (N/P)

Generally, the MW of CS exerts a major influence on its biological and physicochemical characteristics. Studies have shown that siRNA transfection efficiency can be modulated by the MW of CS, and since the MW affects polymer chain entanglement, this in turn influences its complexing ability with negatively charged siRNA. For instance, high MW chitosan will entangle siRNA more readily than low MW chitosan, which results in binding siRNA more efficiently and protecting the condensed siRNA from enzymatic degradation and serum components. In a complementary fashion, the degree of deacetylation and the N/P charge ratio influence the total charge associated with the nitrogen groups of CS, and the overall charge of the CS/siRNA complex and ultimately the transfection efficiency. In this review, we will focus our attention on the effect of these important parameters on the transfection efficiency for siRNA delivery using chitosan.

Katas and Alpar were the first to develop stable nanocomplexes (<500 nm size) of CS/siRNA following the protocols of Lopez-Leon et al. (2005). In that study (Katas and Alpar, 2006), used the ionic gelation method to prepare four CS samples (provided by Protasan Ultrapure, Pronova Biomedical, Norway) having a DD of 86% with an N/P ratio that varied from 1.25 to 15:1. Two each of CS hydrochloride (MWs of 270 and 110 kDa) and CS glutamate (MWs of 470 and 160 kDa) were made to react with either polyethylene oxide or polypropylene oxide in the presence of penta-sodium tripolyphosphate (PSTPP) as an ionic cross-linking agent as shown in Fig. 2. By adjusting the ratio of CS to PSTPP from 4:1 to 6:1, the surface charge on NPs was made to vary from +40 to +60 mV, but after adding siRNA (10 μg) at the CS:PSTPP weight ratio of 6:1, the surface charge dropped to +30 mV. The addition of PSTPP increases surface charge, but after binding with siRNA, the charge drops because of the neutralization of positive surface charge on CS by the negatively charged siRNA.

The results (Katas and Alpar, 2006) displayed in Fig. 3 reflect the influence of MW and N/P ratio on the size of CS NPs. Smaller size NPs are produced with lower MW CS than higher MW CS, and that size increases considerably with increasing N/P ratio. Alternatively, no significant differences are observed in the sizes of siRNA-loaded and placebo NPs. However, pH exerts an effect on particle size (see Fig. 4). As the pH decreases, particle size also decreases due to the increasing number of protonated amine groups that interact strongly with siRNA and PSTPP to reduce the particle size. A higher transfection efficiency of 83% is observed for siRNA adsorbed onto CS glutamate NPs than CS hydrochloride NPs (72%). *In vitro* experiments performed on CHO K1 and HEK 293 cell lines reveal

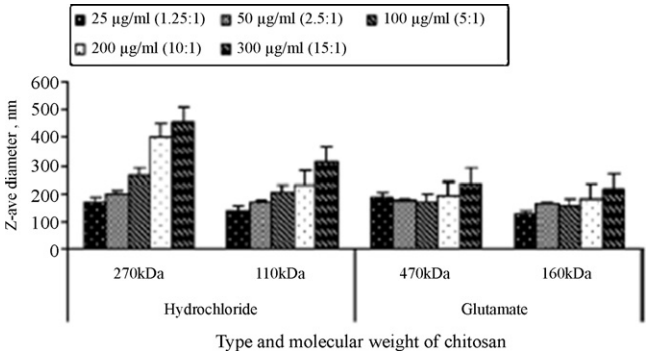

Fig. 3. Effect of MW of CS and N/P ratio on mean particle size of CS/siRNA NPs.

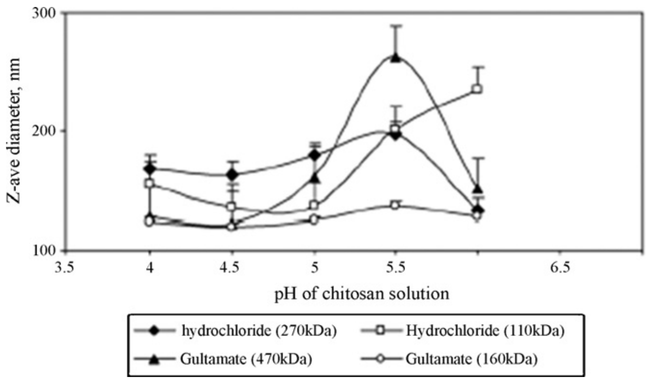

Fig. 4. Effect of pH on mean particle size of CS–PSTPP NPs.

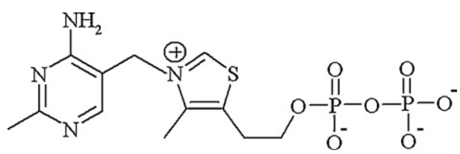

Fig. 5. Chemical structure of thiamine pyrophosphate (TPP).

that gene silencing is dependent on the method of preparation of NPs as well as the MW of CS. For instance, CS–PSTPP/siRNA prepared using PSTPP as a cross-linker are better vectors than CS/siRNA nanocomplexes.

A few studies have shown that thiamine pyrophosphate (TPP) or thiamine diphosphate (see Fig. 5) can be a good cross-linker to prepare NPs of CS, since it is water-soluble and less toxic than PSTPP and plays a key role as a cofactor in carbohydrate metabolism (Engel, 1996) and hence was a prime candidate used to develop CS–siRNA delivery nanocomplexes (Rojanarata et al., 2008). After attaching the phosphate groups of TPP to CS, the water solubility can be improved, since TPP will form a phosphate salt with the protonated amine groups of CS. The amine groups of TPP, particularly at the nitrogen of thiazolium (which is always positive even under physiological pH), will complex with siRNA and thereby, enhance the complex and its transfection efficiency.

Rojanarata et al. in their study provide another example of a case; wherein, a clear-cut dependence of MW on transfection efficiency is observed. Four different CS salts (Seafresh Chitosan Lab., Thailand) were mixed with TPP solution at a molar ratio of 1.5:1.0,

Table 1
Variation of size and zeta potential of CS/siRNA complexes for different values of N/P Howard et al. (2006).

N/P ratio	Size (nm)	Zeta potential (mV)
6	228	18.8
23	319	31.1
71	176	26.8
285	136	29.5

and the nanocomplexes were then prepared by adding siRNA solution to the CS–TPP solution and diluting with 0.1% diethylpyrocarbonate (DEPC). The particle size of NPs prepared from CS of MW 20, 45 and 200 kDa increased with increasing CS–TPP/SiRNA weight ratios from 0.4 to 20, acquiring a maximum size at 20 but showing a decrease once the weight ratio of 40 was reached. In contrast, the nanocomplexes prepared from the CS with a MW of 460 kDa had sizes that varied from 360 to 600 nm at all the weight ratios. The negative zeta potential observed at a weight ratio of 0.4 became positive at a weight ratio of 20, but at greater than 20, the zeta potential increased from +5 to +15 mV as shown in Fig. 6.

The *in vitro* studies of transfection efficiency in stable and endogenous enhanced green fluorescent protein (EGFP) expressing HepG2 and a human hepatocarcinoma cell line showed a high gene-silencing efficiency without showing any influence of pH. Comparatively, a negligible gene-silencing effect was observed for siRNA alone, while, complexes of CS–TPP/siRNA containing EGFP-mismatched siRNA showed no EGFP inhibition, confirming knockdown specificity. The cytotoxicity was evaluated using the MTT (methylthiazolyldiphenyl-tetrazolium bromide) cell proliferation method and TPP/siRNA complexes were found to be safe to use (Smith et al., 1992).

The silencing efficiency of CS–TPP/siRNA complexes increased with increasing N/P giving the highest gene-silencing effect of 70–73% at an N/P ratio of 80 for CS samples with a MW of 20 and 45 kDa (see Fig. 7). Smaller size complexes were more effective than larger ones in reducing gene expression. For formulations with N/P ratios of 40 and 80, the gene-silencing efficiency decreased with increasing MW of CS. The reduction in EGFP expression was also observed in stable constitutive EGFP-HepG2 cells treated with CS–TPP/siRNA complexes after 1–5 days post-transfection. These findings show that both the MW of CS and the N/P ratio of CS–TPP/siRNA complex affect the transfection efficiency of siRNA (Rojanarata et al., 2008).

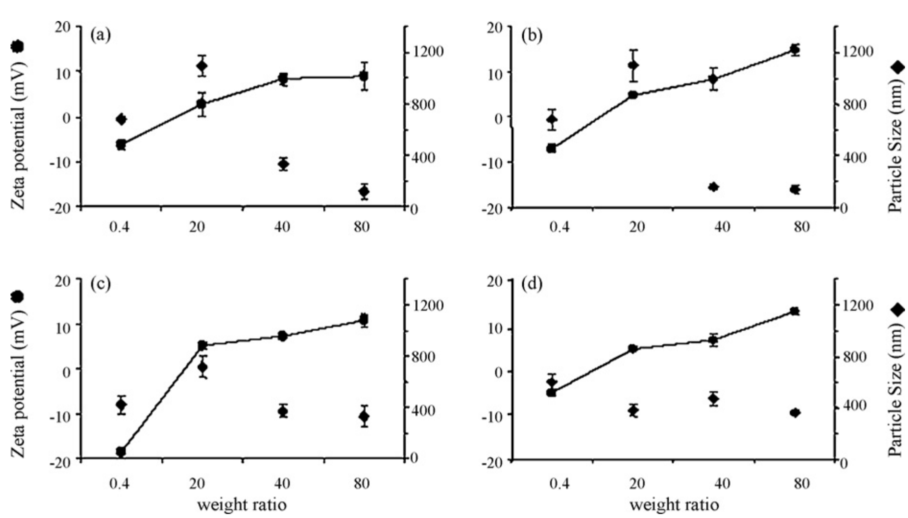

Fig. 6. Variation of zeta potential and particle size with weight ratios of CS–TPP/siRNA complexes for different MWs viz., (a) 20 kDa, (b) 45 kDa, (c) 200 kDa and (d) 460 kDa of chitosan.

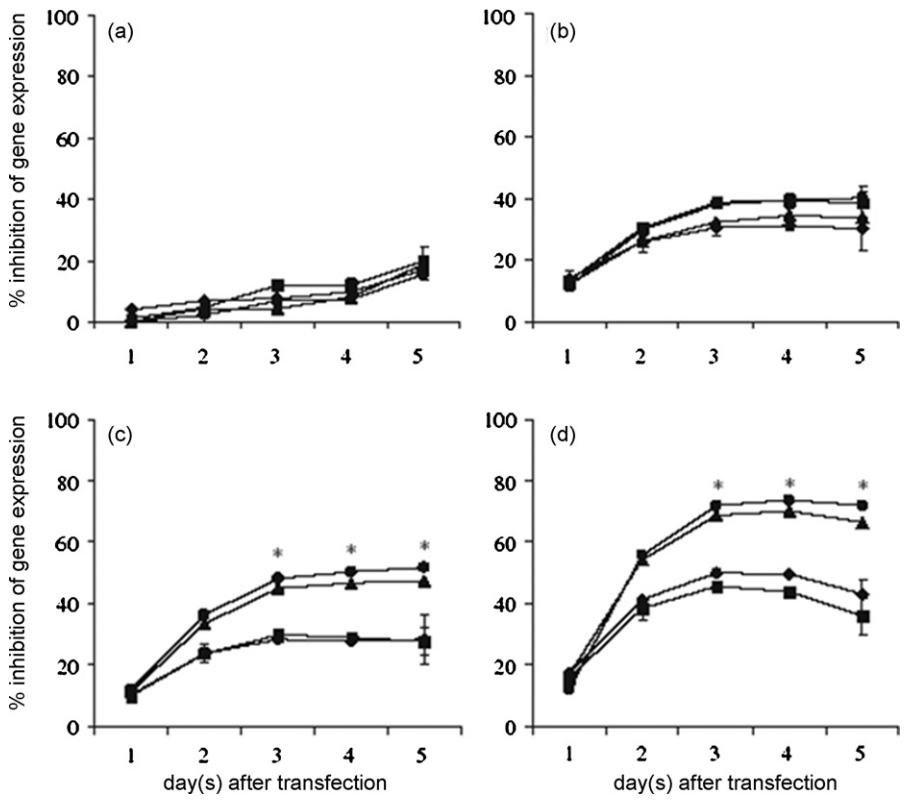

Fig. 7. Effect of weight ratio and MW on gene silencing (% EGFP gene knockdown) of CS–TPP/siRNA complexes in HepG2 cells after 1-5 days post transfection. Different N/P ratios: (a) N/P = 0.4, (b) N/P = 20, (c) N/P = 40, (d) N/P = 80. Symbols for different MW CS samples: 20 kDa (filled circle); 45 kDa (filled triangle); 200 kDa (filled diamond) and 460 kDa (filled square).

Malhotra et al. (2009) studied the effect of MW and N/P ratio on tranfection and gene-silencing efficiency. They developed ultrafine NPs using the ionic gelation method (Calvo et al., 1997) with sodium tripolyphosphate as a cross-linker and three CS samples of different MW (10 100 and 500 kDa; Wako, Richmond, VA, USA) each having a viscosity of 5–20 cP and DD of 80%. Different N/P ratios of CS–TPP ranging from 3:1 to 7:1 were used to produce nanocomplexes of around 20 nm having a positive surface charge of c. 16.5 mV. The pH affected the particle size and surface charge such that at pH 5, the particle size was the smallest irrespective of the MW of CS. With increasing size of NPs, transfection efficiency decreased giving maximum transfection efficiency at the N/P ratio of 200:1. The gene-silencing efficiency studied under varying N/P ratios and pH, performed on mouse neuro-blastoma cells (Neuro2a), and transfection efficiency analyzed by measuring fluorescence intensity suggest that the lowest particle size of 20 nm gives the highest transfection efficiency.

Howard et al. (2006) studied the effect of N/P ratio on size and zeta potential, by performing *in vitro* and *in vivo* experiments on chitosan-based nanocomplexes of CS/siRNA (chitosan of MW 114 kDa, 84% DD; Bioneer A/S, Horsholm, Denmark) in the size range of 40–600 nm. Both the size and the morphology of NPs had a significant effect on *in vivo* studies in human carcinoma cell line and murine peritoneal macrophages expressing EGFP. The results summarized in Table 1 show the dependence of size on N/P ratio, i.e., the higher the N/P ratio, the smaller the particle size (except for values at a low N/P ratio of 6). The formulations possess a net positive charge of greater than 18 mV and after testing on NIH 3T3 cells for over 24 h, *in vivo* testing indicated rapid uptake (1 h) of Cy5-labeled NPs, followed by accumulation over a 24 h period. Knockdown of the endogenous enhanced EGFP was demonstrated in both H1299 human lung carcinoma cells and murine peritoneal macrophages (77.9% and 89.3% reductions in EGFP fluorescence, respectively). Western Blot analysis showed 90% reduced expression of BCR/ABL-1 leukemia fusion protein, while BCR expression was unaffected in K562 (Ph^+) cells after the transfection. Effective *in vivo* RNAi was achieved in bronchiole epithelial cells of transgenic EGFP mice after nasal administration of CS/siRNA formulations (37% and 43% reductions compared to mismatch and untreated control, respectively). Compared to earlier reports on polyethyleneimine-based systems for adherent cell lines (Urban-Klein et al., 2005), the present work showed efficient knockdown.

Liu et al. (2007) prepared CS/siRNA nanoparticles with a 200 nm diameter using CS (Cognis Deutschland GmbH & Co., Dusseldorf, Germany) with a MW range of 50–1000 kDa and DD range of 80–95%. They found that size, zeta potential, morphology and complex stability as well as *in vitro* gene silencing of CS/siRNA nanoparticles were dependent on MW and DD. High MW and high DD samples produced stable NPs, while those prepared with low MW (10 kDa) and an N/P ratio of 50 showed almost no knockdown of endogenous EGFP in H1299 human lung carcinoma cells. On the other hand, NP's prepared from MW's in the range of 65–170 kDa and a DD of 80% showed a gene-silencing efficiency between 45% and 65%. The highest gene-silencing efficiency of 80% was achieved when using an N/P ratio of 150 for MWs of 114 and 170 kDa having a DD of 84%.

A complex coacervation technique was used to produce NPs in the size range of 180–370 nm using CS samples (Sigma–Aldrich, St. Louis, MO, USA) of low MW (viscosities of 20–200 cP) and medium MW (viscosities of 200–800 cP) to encapsulate siRNA (Yuan et al., 2009). AFM experiments on HeLa transfected cells after treatment with siRNA showed a rough surface when compared to normal cells. These results are consistent with those of other investigators (Liu et al., 2007). *In vitro* studies on HeLa cells did not show any dependence of transfection efficiency of CS/SiRNA on the MW of CS. Transfection efficiency in HeLA cells

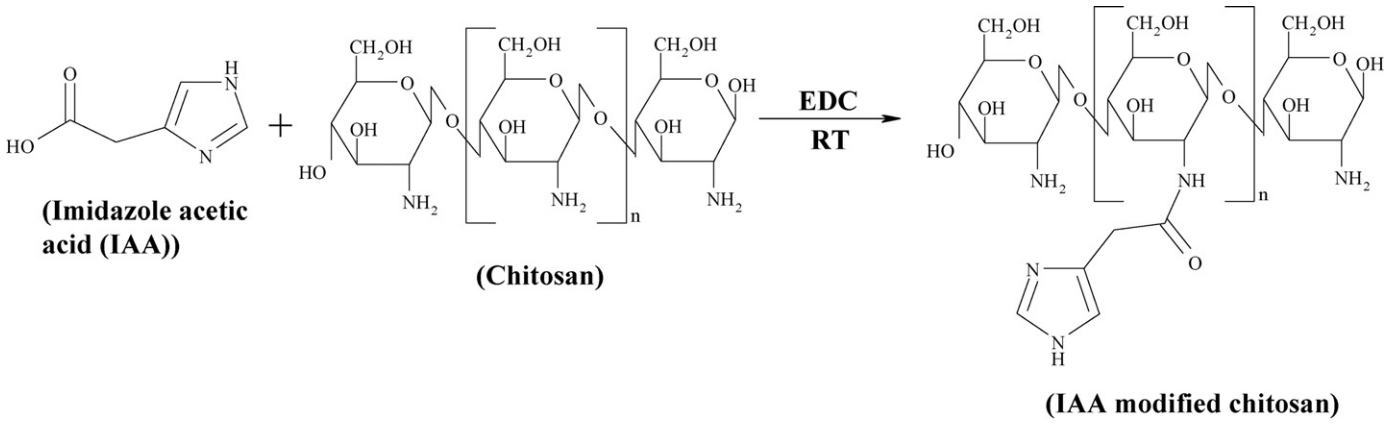

Fig. 8. Structure of IAA modified chitosan.

was measured by counting green fluorescent marker using fluorescent activated cell sorting (FACS), and at a CS concentration of 1 μg/mL, the transfection efficiencies of low and medium MW were 4.47% and 4.18%, respectively, which were higher than those observed for the negative control siRNA (0.04%). By increasing the CS concentration to 15 μg/mL, the intensity of the green fluorescence also increased giving transfection efficiencies of 22% and 32%, respectively for the low and medium MW when compared to the transfection efficiency of the positive control, Lipofectamine™ 2000/siRNA.

Recently, Ji et al. (2009) studied the effect of MW and DD of CS samples ranging in MW from 190 to 310 kDa and a DD of 75–85%. The CS/siRNA NPs were produced with irregular, lamellar and dendritic structures having a size around 148 nm and a zeta potential of 58 mV. The carrier system was used to investigate the knockdown efficiency of gene expression in FHL2 over-expressed human colorectal cancer Lovo cells and achieved a knockdown of 70% FHL2 gene expression. Western blot analysis showed that significant FHL2 protein expression was reduced by the NPs; blocking FHL2 expression by siRNA inhibited the growth and proliferation of human colorectal cancer Lovo cells.

From the foregoing discussions, it is quite evident that molecular weight, degree of deacetylation and N/P charge ratio of the CS–siRNA complexes are major influencing factors in controlling transfection efficiency.

5.2. Nanocomplexes of chitosan derivatives

Chitosan and its chemically modified derivatives have also been used as effective transfection agents for a number of cell lines. In a recent report by Ghosn et al. (2008a,b), chemical modification of CS was done by introducing an imidazole acetic acid moiety to CS via carbodiimide chemistry as shown in Fig. 8. This system showed enhanced endosomal escape and cytoplasmic siRNA dissociation and offered an enhanced gene-silencing effect. The NP formulations at different N/P ratios ranging from 5 to 50 were developed in the size range of 300 nm. The zeta potentials ranged from +15 mV for an N/P of 5 to +38 mV for an N/P of 50. NPs with a size greater than 300 nm demonstrated significant gene knockdown efficiency *in vitro*. The efficiency was nearly twice that of unmodified CS/siRNA in HEK293 cells.

Pille et al. (2006) investigated the efficacy of intravenously administered encapsulated anti-RhoA (Ras homologous A) siRNA in CS-coated polyisohexylcyanoacrylate (PIHCA) NPs toward xenografted aggressive breast cancers (MDA-MB-231). After administering the NP's for 3 days at a dose rate of 150 or 1500 μg/kg body weight of the mice, the growth of tumors was inhibited by 90% in the 150 μg/kg animal models and even more in the 1500 μg/kg group. This therapy using anti-RhoA siRNA was effective in cancer treatment.

Anderson et al. (2008) devised a method to produce gene silencing, active, lyophilized, cationic CS or lipid (*Trans*IT-TKO) siRNA formulations for efficient knockdown of EGFP in H1299 human lung carcinoma cells. The cells were transfected in plates pre-coated with both *Trans*IT-TKO/siRNA (85%) and CS/siRNA formulations containing sucrose as a lyoprotectant (70%). The nanocomplexes produced were in the size range of 126–168 nm (the latter with 10% sucrose) by a modified technique described previously (Ghosn et al., 2008a). Both the systems were lyophilized onto microwell plastic plates with and without excipients. The cellular toxicity and knockdown efficiency of EGFP expressed in human epithelial cell line H1299 and cytokine tumor necrosis factor (TNF-α) in the RAW 246.7 murine macrophage cell line were quite effective in achieving gene silencing. The advantage of the Anderson method is that it avoids siRNA reconstitution before adding onto cells. The silencing activity of CS/siRNA was active for two months when stored at ambient temperature. Silencing of pro-inflammatory TNF-α was also demonstrated in RAW macrophage cell line using lyophilized CS/siRNA, suggesting that the coating has improved the biocompatibility.

Even though conventional organic fluorophores have been used to track the delivery of siRNA *in vitro*, they do not match the superior optical properties of quantum dots (QDs). QDs are colloidal semiconductor nanocrystals of only a few nanometer size that exhibit unique optical and electronic properties due to quantum confinement effects. Compared to conventional organic fluorophores, QDs are more stable toward photo-bleaching, have tighter and more controllable emission bands, broader absorption spectra and higher quantum yields. Thus, instead of using conventional fluorophores, QDs in CS NPs were prepared in order to provide a more attractive way of monitoring the delivery of siRNA into cells, especially when long-term tracking is required (Tan et al., 2007). They prepared NPs of CS loaded with human epidermal growth factor receptor 2 (HER2siRNA) and doped with fluorescent QDs as a self-tracking and non-viral vehicle to deliver siRNA. Water-soluble green fluorescent CdSe/ZnS QDs were synthesized as described before (Yang and Zhang, 2004) and used to bind to cationic CS through an electrostatic attraction between oppositely charged species, forming monodisperse CS–QD NPs of around 60 nm. The size of the NPs even after conjugation with siRNA was around 80 nm with a negative zeta potential. The N/P ratio of 8 was found to be optimal for conjugation of HER2siRNA to CS–QDs. The NPs successfully tracked the *in vitro* delivery of HER2siRNA (Ghosn et al., 2008a), and they were readily internalized into cells thus, achieving a desirable silencing effect on HER2 gene via RNAi.

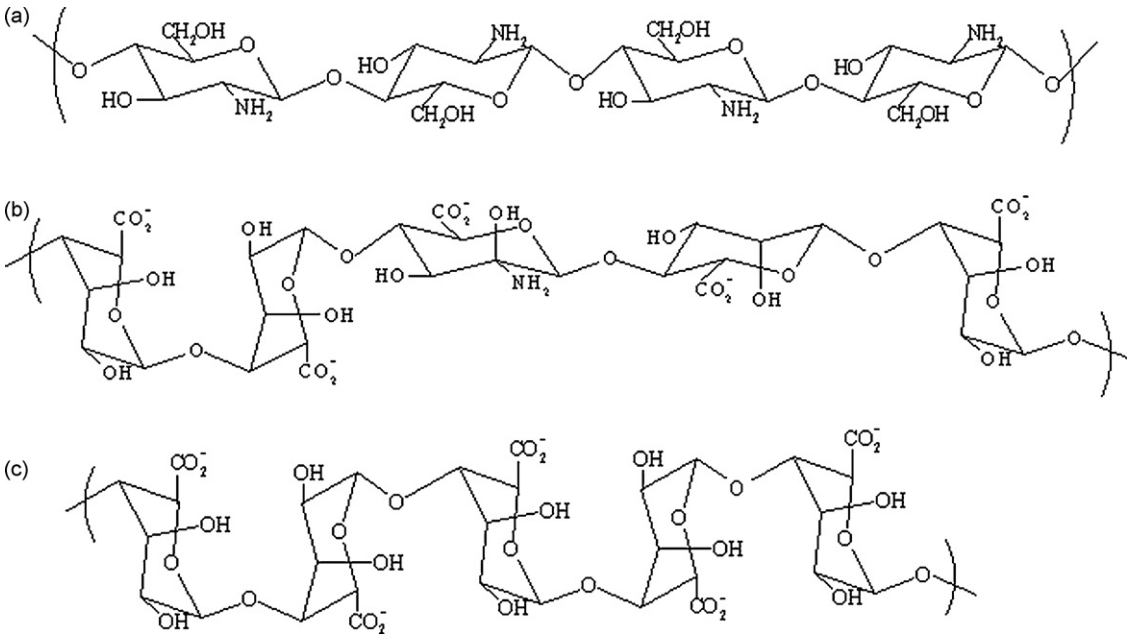

Fig. 9. Chemical structures of (a) chitosan, (b) alginate and (c) polyguluronate (PG).

Tahara et al. (2008) developed a simple method to prepare CS-coated PLGA NPs for gene delivery using the emulsion solvent diffusion (ESD) method without sonication, by dissolving both polymer and drug in organic solvent. DOTAP was used as a cationic agent to increase the loading efficiency of siRNA. PLGA NPs coated with CS reduced the initial burst of siRNA release, but prolonged its release at a later stage. The CS coating also enhanced the transport of siRNA across the cell membrane. Particle size, zeta potential, drug loading efficiency and the release profile of siRNA-loaded and CS-coated PLGA NPs prepared by the ESD method were compared with those NPs prepared by the water/oil/water (w/o/w) emulsion solvent evaporation method and found to be better.

Lee et al. (2009) reported a potential siRNA delivery system using CS and an alginate derivative viz., polyguluronate (PG), which is biocompatible and nontoxic, and can be used to crosslink CS to form stable NPs when prepared by a coacervation method (see Fig. 9). Various physicochemical properties of the CS/PG NPs, including size, surface charge, morphology and interaction with siRNA were studied. CS with a MW of 470 kDa and a DD of 86% was used, while the alginate had a MW of 200 kDa (both from

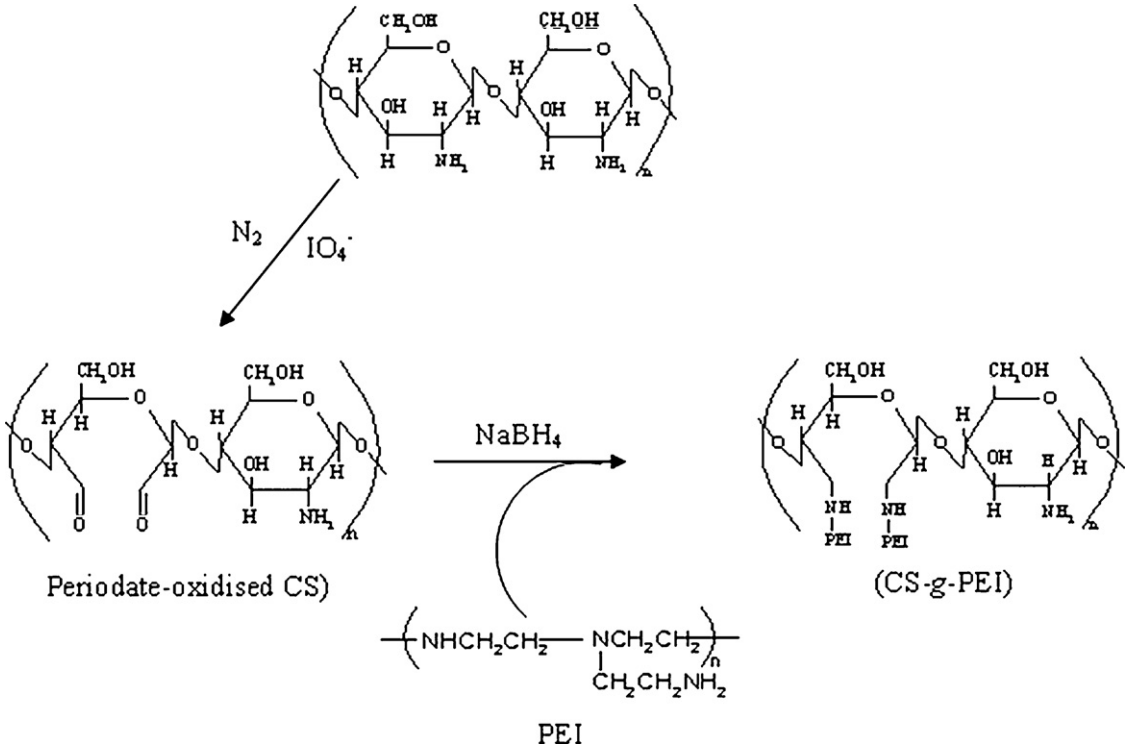

Fig. 10. Reaction scheme used in the synthesis of CS-g-PEI.

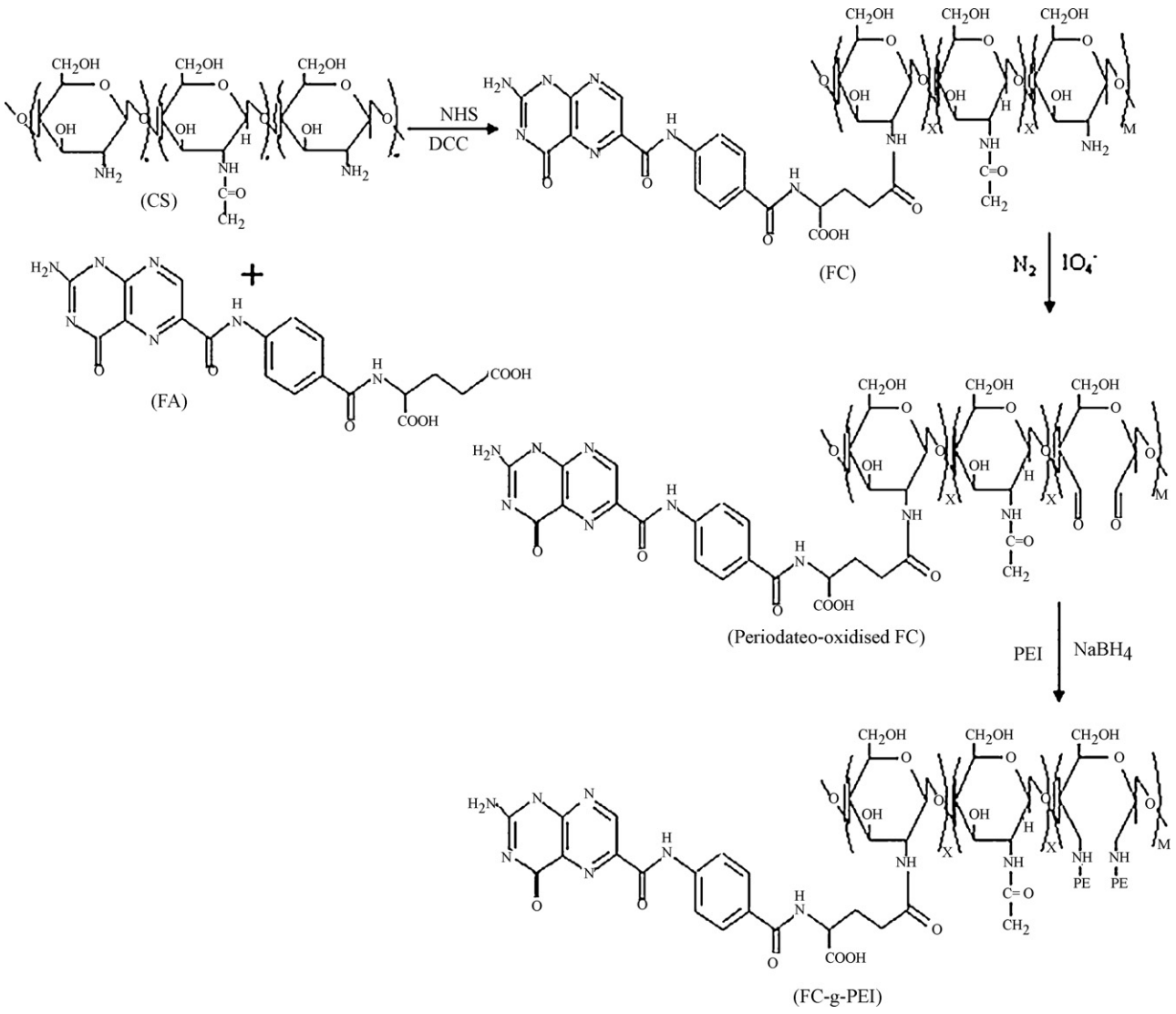

Fig. 11. Reaction scheme used in the synthesis of folate-CS-g-PEI copolymer.

FMC Biopolymer, Norway). The size of the nanoparticles ranged from 110 to 430 nm, as the N/P ratio increased from 5 to 20 and the surface charge remained around +10 mV. The CS/PG loaded with siRNA showed low toxicity and a gene-silencing effect in the presence of serum when delivered to HEK 293FT and HeLa cells; Lipofectamine and poly(L-lysine) were used as positive controls. The gene-silencing effect was negligible for naked siRNA, while complexes of Lipofectamine/mismatch siRNA demonstrated knockdown specificity, indicating that the gene-silencing effect was enhanced by the inclusion of PG.

Jere et al. (2009a,b) developed CS-graft-polyethyleneimine (CS-g-PEI) copolymer composed of CS with a MW of 100 kDa and a DD of 88% and low molecular weight PEI for delivering siRNA to lung cancer cells. Synthesis of CS-g-PEI was done through an imine reaction between periodate-oxidized CS and low MW PEI (1.8 kDa) as described previously (Jiang et al., 2007) (see Fig. 10). The MW of the copolymer was around 25 kDa and the NP size 150 nm. The CS-g-PEI complex loaded with siRNA was stable with a compact spherical morphology, and used for the silencing of reporter EGFP and oncoprotein Akt1. The consequences of Akt1 silencing on cancer cell survival, proliferation, malignancy and metastasis were evaluated *in vitro*. These nanocomplexes, when delivered to the cells, efficiently reduced Akt1 protein expression in A549 cells, suggesting that the carrier has a tremendous potential in siRNA-based cancer studies, since it can be safely and efficiently delivered to lung cancer cells.

In a continuing study by Jiang et al. (2009), folate-CS-g-PEI copolymer was prepared by an imine reaction between periodate-oxidized folate-CS (FCS) and low MW PEI. The nanocomplex was developed as a siRNA carrier for cancer cell targeting (see Fig. 11). The size of the NP's was less than 80 nm. At N/P = 0.1, where the complexes could not be formed completely, the zeta potential was negative (−23 mV), but with increasing N/P ratios, the zeta potential rapidly increased to positive values (18 mV at N/P = 14). Cytotoxicity, transfection efficiency and cancer cell specificity of these systems were better than the previously reported CS-g-PEI carrier device (Jere et al., 2009a,b). Aerosol delivery of folate-CS-g-PEI/Akt1 siRNA suppressed lung tumorigenesis in the urethane-induced lung cancer mouse model through an Akt signaling pathway.

6. *In vitro* and *in vivo* applications of CS/siRNA complexes

From the foregoing discussions, it is evident that CS and CS-based devices are safe and efficient in gene delivery. However, cancer treatment is by far the most important proposed applica-

tion of gene therapy and many clinical trials using gene therapy are under investigation. Particularly, cationic polymers including CS derivatives have been used in animal models, but clinical trials are lagging due to a low transfection efficiency. Even though the formation of CS–siRNA nanoparticles is a vital requirement, the stability and extra cellular protection of siRNA is equally important. After cellular uptake of stable NPs by endocytosis, endosome lysis and disassembly are needed to allow RNA mediated gene silencing. Thus, a proper balance between protection and release plays a crucial role in the biological functioning of siRNA (Kim et al., 2007).

Compared to research *in vitro* with CS-based systems, *in vivo* research is still in the developmental stage. Only a few studies are available which detail *in vivo* trials of CS–siRNA nanocomplexes in silencing gene expression in animals. As discussed before, Howard et al. (2006) used CS–siRNA nanocomplexes for silencing EGFP expression in the transgenic EGFP mouse model. Zhang et al. (2005) investigated Nanogene 042 for *de novo* expression of siNS1 in lung tissues for protection from RSV infection and delivered the plasmid-born siRNA targeting the NS1 gene (siNS1). The Nanogene 042 showed higher transfection efficiency and induced lesser inflammation than the classical high MW chitosan. Pille et al. (Pille et al., 2006) reported the successful administration of anti-RhoA siRNA by CS-coated poly(isohexyl cyanoacrylate) nanoparticles intravenously, which is essential for the success of siRNA technology in clinical strategies and also advantageous for targeting primary tumors.

7. Conclusions

RNA interference has proven to be an extremely potent and versatile tool to specifically knockdown the expression of targeted genes. In recent years, this technology is emerging as a powerful tool for *in vivo* research, both to address questions of basic biology and the needs of drug development programs. From the literature, it is evident that chitosan or its derivatives are promising carriers, since the polymers are non-toxic, biodegradable and less immunogenic than viral vectors. Their successful application, however, depends on optimizing molecular weight, degree of deacetylation, the chitosan/siRNA ratio (N/P ratio), pH, and the type and nature of any derivatizing or cross-linking agent. A low molecular weight, high degree of deacetylation, small particle size (100 nm) and a moderate, positive, surface zeta potential along with a high N/P ratio are advantageous to achieve high transfection efficiency. Among non-viral vectors for siRNA delivery, chitosan and its derivatives are promising alternatives to viral vectors for targeting siRNA to specific cells.

Acknowledgements

Tejraj M. Aminabhavi thanks CSIR [21(0760)/09/EMR-II], New Delhi, India for awarding Emeritus Scientist status. Walter E. Rudzinski would like to thank both Research Corporation (grant 6576) and the Welch Foundation (grant AI0045) for partial support of this work.

References

Agaard, L., Rossi, J.J., 2007. RNAi therapeutics: principles, prospects and challenges. Adv. Drug Deliv. Rev. 59, 75–86.
Agnihotri, S.A., Mallikarjuna, N.N., Aminabhavi, T.M., 2004a. Recent advances on chitosan-based micro- and nanoparticles in drug delivery. J. Control. Release 100, 5–28.
Agnihotri, S.A., Aminabhavi, T.M., 2004b. Controlled release of clozapine through chitosan microparticles prepared by a novel method. J. Control. Release 96, 245–259.
Akhtar, S., Benter, I.F., 2007. Nonviral delivery of synthetic siRNAs in vivo. J. Clin. Invest. 117, 3623–3632.
Anderson, M.O., Howard, K.A., Paludan, S.R., Besenbacher, F., Kjems, J., 2008. Delivery of siRNA from lyphilized polymeric surfaces. Biomaterials 29, 506–512.
Behlke, M.A., 2006. Progress towards *in vivo* use of siRNAs. Mol. Ther. 13, 644–670.
Borchard, G., 2001. Chitosans for gene delivery. Adv. Drug Deliv. Rev. 52, 145–150.
Calvo, P., Remunan-lopez, C., Villa-Jato, J.L., Alonso, M.J., 1997. Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers. J. Appl. Polym. Sci. 63, 125–132.
Couzin, J., 2002. Small RNAs make big splash. Science 298, 2296–2297.
Dean, D.A., 2000. Peptide nucleic acids: versatile tools for gene therapy strategies. Adv. Drug Deliv. Rev. 44, 81–95.
Deshayes, S., Morris, M., Heitz, F., Divita, G., 2008. Delivery of proteins and nucleic acids using a non-covalent peptide-based strategy. Adv. Drug Deliv. Rev. 60, 537–547.
Engel, P.C., 1996. Enzyme cofactors. In: Engel, P.C. (Ed.), Enzymology LabFax. Academic, California, pp. 244–245.
Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M., Danielsen, M., 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. U.S.A. 84, 7413–7417.
Fernando, S., Fletcher, B.S., 2006. Sleeping beauty transposon-mediated nonviral gene therapy. Biodrugs 20, 219–229.
Fillion, M.C., Philips, N.C., 1997. Major limitations in the use of cationic liposomes for DNA delivery. Int. J. Pharm. 162, 159–170.
Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double stranded RNA in Caenorhabditis elegans. Nature 391, 806–811.
Florea, B.I., Thanou, M., Junginger, H.E., Borchard, G., 2006. Enhancement of bronchial octreotide absorption by chitosan and N-trimethyl chitosan shows linear *in vitro*/*in vivo* correlation. J. Control. Release 110, 353–361.
Flynn, M.A., Casey, D.G., Todryk, S.M., Mahon, B.P., 2004. Efficient delivery of small interfering RNA for inhibition of IL-12p40 expression *in vivo*. J. Inflammat. (Lond.) 1, 4.
Garnett, M.C., 1999. Gene delivery systems using cationic polymers. Crit. Rev. Ther. Drug Carrier Syst. 16, 147–207.
Gao, S.Y., Chen, J.N., Dong, L., Ding, Z., Yang, Y.H., Zhang, J.F., 2005. Targeting delivery of oligonucleotide and plasmid DNA to hepatocyte via galactosylated chitosan vector. Eur. J. Pharm. Biopharm. 60, 327–334.
Gary, D.J., Puri, N., Won, Y.-Y., 2007. Polymer-based siRNA delivery: perspectives on the fundamental and phenomenological distinctions from polymer-based DNA delivery. J. Control. Release 121, 64–73.
Ghosn, B., Singh, A., Roy, K., 2008a. Efficient siRNA delivery by secondary and tertiary amine modified polysaccharides. NSTI-Nanotech 2, 338–341.
Ghosn, B., Kasturi, S.P., Roy, K., 2008b. Enhancing polysaccharide-mediated delivery of nucleic acids through functionalization with secondary and tertiary amines. Curr. Top. Med. Chem. 8, 331–340.
Gleave, M.E., Monia, B.P., 2005. Antisense therapy for cancer. Nat Rev. Cancer 5, 468–479.
Howard, K.A., Rahbek, U.L., Liu, X.D., Damgaard, C.K., Glud, S.Z., Andersen, M.O., Hovgaard, M.B., Schmitz, A., Nyengaard, J.R., Besenbacher, F., Kjems, J., 2006. RNA interference *in vitro* and *in vivo* using a chitosan/siRNA nanoparticle system. Mol. Ther. 14, 476–484.
Ikeda, Y., Taira, K., 2006. Ligand-targeted delivery of therapeutic siRNA. Pharm. Res. 23, 1631–1640.
Illum, L., 1998. Chitosan and its uses as a pharmaceutical excipient. Pharm. Res. 15, 1326–1331.
Il'ina, A.V., Varlamov, V.P., 2005. Chitosan-based polyelectrolyte complexes: a review. Appl. Biochem. Microbiol. 41, 5–11.
Jere, D., Jiang, H.-L., Kim, Y.-K., Arote, R., Choi, Y.-J., Yun, C.-H., Cho, M.-H., Cho, C.-S., 2009a. Chitosan-graft-polyethylenimine for Akt1 siRNA delivery to lung cancer cells. Int. J. Pharm. 378, 194–200.
Jere, D., Jiang, H.-L., Kim, Y.-K., Arote, R., Choi, Y.-J., Yun, C.-H., Cho, M.-H., Cho, C.-S., 2009b. Degradable polyethylenimines as DNA and small interfering RNA carriers. Expert Opin. 6, 827–833.
Ji, A.M., Su, D., Che, O., Li, W.S., Sun, L., Zhang, Z.Y., Yang, B., Xu, F., 2009. Functional gene silencing mediated by chitosan/siRNA nanocomplexes. Nanotechnology 20, 405–412.
Jiang, H.L., Kim, Y.K., Arote, R., Nah, J.W., Cho, M.H., Choi, Y.J., Akaike, T., Cho, C.S., 2007. Chitosan-graft-polyethyleneimine as a gene carrier. J. Control. Release 117, 273–280.
Jiang, H.-L., Xu, C.-X., Kim, Y.-K., Arote, R., Jere, D., Lim, H.-T., Cho, M.-H., Cho, C.-S., 2009. The suppression of lung tumorigenesis by aerosol-delivered folate-chitosan-graft-polyethylenimine/Akt1 siRNA complexes through the Akt signaling pathway. Biomaterials 30, 5844–5852.
Katas, H., Alpar, H.O., 2006. Development and characterization of chitosan nanoparticles for siRNA delivery. J. Control. Release 115, 216–225.
Kiang, T., Wen, J., Lim, H.W., Leong, K.W., 2004. The effect of the degree of chitosan deacetylation on the efficiency of gene transfection. Biomaterials 25, 5293–5301.
Kihara, F., Arima, H., Tsutsumi, T., Hirayama, F., Uekama, K., 2002. Effects of structure of polyamidoamine dendrimer on gene transfer efficiency of the dendrimer conjugate with α-cyclodextrin. Bioconjug. Chem. 13, 1211–1219.
Kihara, F., Arima, H., Tsutsumi, T., Hirayama, F., Uekama, K., 2003. In vitro and in vivo gene transfer by an optimized α-cyclodextrin conjugate with polyamidoamine dendrimer. Bioconjug. Chem. 14, 342–350.
Kim, T.-H., Jiang, H.-L., Jere, D., Park, I.-K., Cho, M.-H., Nah, J.-W., Choi, Y.-J., Akaike, T., Cho, C.-S., 2007. Chemical modification of chitosan as a gene carrier in vitro and in vivo. Prog. Polym. Sci. 32, 726–753.

Lee, D., Zhang, W., Shirley, S.A., Kong, X., Hellermann, G.R., Lockey, R.F., Mohapatra, S.S., 2007. Thiolated chitosan DNA/nanocomplexes exhibit enhanced and sustained gene delivery. Pharm. Res. 24, 157–167.
Lee, D.W., Yun, K.-S., Ban, H.-S., Choe, W., Lee, S.K., Lee, K.Y., 2009. Preparation and characterization of chitosan/polyguluronate nanoparticles for siRNA delivery. J. Control. Release 139, 146–152.
Leong, K.W., 2005. Polymeric controlled nucleic acid delivery. MRS Bull. 30, 640–646.
Liu, W., Sun, S., Cao, Z., Zhang, X., Yao, K., Lu, W.W., Luk, K.D., 2005. An investigation on the physicochemical properties of chitosan/DNA polyelectrolyte complexes. Biomaterials 26, 2705–2711.
Liu, X.D., Howard, K.A., Dong, M.D., Andersen, M.O., Rahbek, U.L., Johnsen, M.G., Hansen, O.C., Besenbacher, F., Kjems, J., 2007. The influence of polymeric properties on chitosan/siRNA nanoparticle formation and gene silencing. Biomaterials 28, 1280–1288.
Lopez-Leon, T., Carvalho, E.L.S., Seijo, B., Ortega-Venuesa, J.L., Bastos-Gonzalez, D., 2005. Physicochemical characterization of chitosan nanoparticles: electrokinetic and stability behavior. J. Colloid Interface Sci. 283, 344–351.
Luo, D., Saltzman, W.M., 2000. Enhancement of transfection by physical concentration of DNA at the cell surface. Nat. Biotechnol. 18, 893–895.
Malhotra, M., Kulamarva, A., Sebak, S., Paul, A., Bhathena, J., Mirzaei, M., Prakash, S., 2009. Ultrafine chitosan nanoparticles as an efficient nucleic acid delivery system targeting neuronal cells. Drug Dev. Ind. Pharm. 35, 719–726.
Mansouri, S., Lavigne, P., Corsi, K., Benderdour, M., Beaumont, E., Fernandes, J.C., 2004. Chitosan-DNA nanoparticles as non-viral vectors in gene therapy: strategies to improve transfection efficacy. Eur. J. Pharm. Biopharm. 57, 1–8.
Mao, H.Q., Roy, K., Troung-Le, V.L., Janes, K.A., Lin, K.Y., Wang, Y., August, J.T., Leong, K.W., 2001. Chitosan-DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency. J. Control. Release 70, 399–421.
Nielsen, P.E., 2001. Targeting double stranded DNA with peptide nucleic acid (PNA). Curr. Med. Chem. 8, 545–550.
Pack, D.W., Hoffman, A.S., Pun, S., Stayton, P.S., 2005. Design and development of polymers for gene delivery. Nat. Rev. Drug Discov. 4, 581–593.
Park, I.K., Kim, T.H., Park, Y.H., Shin, B.A., Choi, E.S., Chowdhury, E.H., Akaike, T., Cho, C.S., 2001. Galactosylated chitosan-graft-polyethylene glycol as hepatocyte-targeting DNA carrier. J. Control. Release 76, 349–362.
Pille, J.Y., Li, H., Blot, E., Bertrand, J.R., Pritchard, L.L., Opolon, P., Maksimenko, A., Lu, H., Vannier, J.P., Soria, J., Malvy, C., Soria, C., 2006. Intravenous delivery of anti-RhoA small interfering RNA loaded in nanoparticles of chitosan in mice: safety and efficacy in xenografted aggressive breast cancer. Hum. Gene Ther. 17, 1019–1026.
Rajesh, M., Sen, J., Srujan, M., Mukherjee, K., Sreedhar, B., Chaudhari, A., 2007. Dramatic influence of the orientation of linker between hydrophilic and hydrophobic lipid moiety in liposomal gene delivery. J. Am. Chem. Soc. 129, 11408–11420.
Rao, N.M., Gopal, V., 2006a. Cationic lipids for gene delivery in vitro and in vivo. Exp. Opin. Ther. Pat. 16, 825–844.
Rao, N.M., Gopal, V., 2006b. Cell biological and biophysical aspects of lipid-mediated gene delivery. Biosci. Rep. 26, 301–324.
Rojanarata, T., Opanasopit, P., Techaarpornkul, S., Ngawhirunpat, T., Ruktanonchai, U., 2008. Chitosan-thiamine pyrophosphate as a novel carrier for siRNA delivery. Pharm. Res. 25, 2807–2814.
Romoren, K., Pedersen, S., Smistad, G., Evensen, O., Thu, B.J., 2003. The influence of formulation variables on *in vivo* transfection efficiency and physicochemical properties of chitosan-based polyplexes. Int. J. Pharm. 261, 115–127.
Sato, T., Ishii, T., Okahata, Y., 2001. In vitro gene delivery mediated by chitosan: effect of pH, serum, and molecular mass of chitosan on the transfection efficiency. Biomaterials 22, 2075–2080.
Scales, C.W., Huang, F., Li, N., Vasilieva, Y.A., Ray, J., Convertine, A.J., McCormick, C.L., 2006. Corona-stabilized interpolyelectrolyte complexes of siRNA with non-immunogenic, hydrophilic/cationic block copolymers prepared by aqueous RAFT polymerization. Macromolecules 39, 6871–6881.
Smith, M.D., Barbenel, J.C., Courtney, J.M., Grant, M.H., 1992. Novel quantitative methods for the determination of biomaterial cytotoxicity. Int. J. Artif. Organs 15, 191–194.
Tahara, K., Sakai, T., Yamamoto, H., Takeuchi, H., Kawashima, Y., 2008. Establishing chitosan coated PLGA nanosphere platform loaded with wide variety of nucleic acid by complexation with cationic compound for gene delivery. Int. J. Pharm. 354, 210–216.
Takeshita, F., Ochiya, T., 2006. Therapeutic potential of RNA interference against cancer. Cancer Sci. 97, 689–696.
Tan, W.B., Jiang, S., Zhang, Y., 2007. Quantum-dot based nanoparticles for targeted silencing of HER2/neu gene via RNA interference. Biomaterials 28, 1565–1571.
Urban-Klein, B., Werth, S., Abuhurbeid, S., Czubayko, F., Aigner, A., 2005. RNAi-mediated gene-targeting through systemic application of polyethyleneimine (PEI)-complexed siRNA in vivo. Gene Ther. 12, 461–466.
Vaysse, L., Burgelin, I., Merlio, J.P., Arveller, B., 2000. Improved transfection using epithelial cell line-selected ligands and fusegenic peptides. Biochim. Biophys. Acta 1475, 369–376.
Verma, U.N., Surabhi, R.M., Schmaltieg, A., Becerra, C., Gaynor, R.B., 2003. Small interfering RNAs directed against beta-catenin inhibit the in vitro and in vivo growth of colon cancer cells. Clin. Cancer Res. 9, 1291–1300.
Wacheck, V., Zangemeister-Wittke, U., 2006. Antisense molecules for targeted cancer therapy. Crit. Rev. Oncol. Hemat. 59, 65–73.
Weecharangsan, W., Opanasopit, P., Ngawhirunpat, T., Apirakaramwong, A., Rojanarata, T., Ruktanonchai, U., Lee, R.J., 2008. Evaluation of chitosan salts as non-viral gene vectors in CHO-K1 cells. Int. J. Pharm. 348, 161–168.
Xu, S., Dong, M.D., Liu, X.D., Howard, K.A., Kjems, J., Besenbacher, F., 2007. Direct force measurements between siRNA and chitosan molecules using force spectroscopy. Biophys. J. 93, 952–959.
Yang, X.T., Zhang, Y., 2004. Encapsulation of quantum nanodots in polystyrene and silica micro-/nanoparticles. Langmuir 20, 6071–6073.
Yuan, Y.J., Tan, J.Y., Wang, Y.F., Qian, C.W., Zhang, M.Y., 2009. Chitosan nanoparticles as non-viral gene delivery vehicles based on atomic force microscopy study. Acta Biochim. Biophys. Sin. 41, 515–526.
Yun, Y.H., Jiang, H., Chan, R., Chen, W., 2005. J. Biomater. Sci. Polym. Ed. 16, 1359–1378.
Zhang, S.B., Zhao, B., Jiang, H.M., Wang, B., Ma, B., 2007a. Cationic lipids and polymers mediated vectors for delivery of siRNA. J. Control. Release 123, 1–10.
Zhang, Y., Chen, J., Zhang, Y., Pan, Y., Zhao, L., et al., 2007b. A novel PEGylation of chitosan nanoparticles for gene delivery. Biotechnol. Appl. Biochem. 46, 197–204.
Zhang, W., Yang, H., Kong, X., Mohapatra, S., San Juan-Vergara, H., Hellermann, G., et al., 2005. Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene. Nat. Med. 11, 56–62.